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Endorectal power Doppler ultrasonography is a reliable method for evaluation of rectal cancer angiogenesis

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Abstract. – OBJECTIVE: We aimed to assess the preoperative rectal cancer angiogenesis with Endorectal Power Doppler Ultrasonography by using the Power Doppler Vascularity Index (PDVI) calculated by imaging analysis software, and to compare it with the microvessel density (MVD) in surgical specimens

PATIENTS AND METHODS: This study included 110 patients (39 females; mean age 61.5 years) with rectal cancer. Immunohistochemical staining of surgical specimens with anti-CD-31 antibody was used for MVD evaluation. The PDVI of each tumor was calculated using Endorectal Power Doppler with computer-assisted quantification of colour pixels.

RESULTS: Mean MVD - 163 ± 69 microvessels/mm² (50-328) was used as a cutoff point, differentiating two groups of tumors with high (> 160 mm2) and low (≤ 160 mm²) angiogenic activity. Mean PDVI of 8.9 ± 6.0% (0-27.3) was used as a cutoff point, dividing two groups of tumors with high (> 8%) and low (≤ 8%) PDVI. The MVD and the PDVI showed a good positive correlation (r = 0.438, $p = 0.002$). Patients with low PDVI had 25 months longer overall survival ($p < 0.05$) than patients with high PDVI. Patients with low MVD had 36 months longer survival ($p < 0.05$).

CONCLUSIONS: Endorectal Power Doppler Ultrasonography is a reliable and noninvasive method for assessment of the extent of rectal cancer angiogenesis. Tumor angiogenesis assessed by the PDVI correlated with histological MVD determination and could predict survival rates. Endorectal Power Doppler examination is a useful and reproducible method for *in vivo* preoperative quantitative assessment of tumor vascularization.

Key Words:
Rectal cancer, Angiogenesis, Ultrasonography, Power Doppler, Microvessel density.

Introduction

Individual clinical outcomes of patients with rectal cancer cannot be completely predicted by common prognostic factors such as lymph node involvement, local extension of the disease, etc.[1,2]. In order to improve clinical care and provide the optimal treatment, other prognostic markers must be identified. The extent of tumor angiogenesis plays a key role in tumor growth, invasion and recurrence[3]. The conventional way for assessment of rectal cancer angiogenesis requires either biopsy or a tissue specimen and specific immunohistochemical or molecular biological tests. The microvessel density (MVD) determination is a "gold standard" in the evaluation of tumor neovascularization[4]. MVD assessment is not sufficient in detecting tumor angiogenesis. It has the following drawbacks: invasiveness, variations in the used methodology and tissue samples, which represent just a certain area within the tumor. Therefore, rapid and noninvasive tests that are simple, sensitive and specific are needed to predict prognosis and survival of patients with rectal cancer. Noninvasive imaging procedures such as Perfusion Computed Tomography (CT), Dynamic Contrast-enhanced Magnetic Resonance (DCE - MR) Perfusion, and Contrast-enhanced Ultrasound (CEUS) are enforced to observe tumor vascularity[5,6]. However, none of these imaging technologies have entered into routine clinical practice. On the other hand, Doppler Ultrasonography is an appealing method for angiogenesis *in vivo* evaluation, which can be frequently repeated without any risk to the patient[7-10]. However, Color Doppler signals observed within the tumor depict

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vessels with a size of 100 μm or more in diameter. Tumor vessel depicting better than that is needed for more sensitive evaluation of rectal cancer angiogenesis. For instance, Power Doppler Ultasonography is a more suitable modality for depicting the vessels within the tumor due to its high sensitivity in measuring slow flows and its ability to give detailed information about curved and irregular vessels[11]. We suggested that the amount of detected intratumoral vessels correlates with the degree of the tumor's microvascularization. Tumor angiogenesis is not routinely determined in clinical practice, presumably because of the lack of accurate technique for its assessment [12]. The reason for this is the visual subjectivity in the evaluation of vascularization. Consequently, we applied digital assessment of tumor vascularization by using the Computer-assisted Endosonographic method based on the Power Doppler. This was called the Power Doppler Vascularity Index (PDVI). In this study we aimed to assess the preoperative rectal cancer angiogenesis with Endorectal Power Doppler Ultrasonography by using the PDVI calculated by imaging analysis software and to compare it with the MVD in surgical specimens.

Patients and Methods

Patients

This is a prospective observational study. We recruited 110 patients; 39 of which were female and 71 of which were male at an average age of 61.5 ± 11 (27-83) years with rectal cancer. In all the patients, rectal cancer angiogenesis was evaluated by Power Doppler Endosonography, Computer-assisted Power Doppler examination and immunohistochemistry. Then, all the patients were operated and staged according to the World Health Organization (WHO) criteria for colon and rectum as follows: 20 patients (18%) in stage I, 29 patients (26%) in stage II, 47 patients (43%) in stage III and 14 patients (13%) in stage IV[13]. Patients were followed clinically for a mean period of 30.4 ± 17.6 months (6-82) after the operation.

Angiogenesis Evaluation by Power Doppler Endosonography

Power Doppler Endosonography was performed by a Toshiba, Nemio SSA 550A (Tokyo, Japan) ultrasound system with a biplane convex transversal and an end-fire scanning probe PVM-740RT (5.0/7.5/10 MHz/144°) capable of Power Doppler. Enema was given to each patient two hours before the endosonography. The probe was inserted 12-15 cm in the left lateral position and then pulled out to the level of the tumor. Power Doppler settings were regulated to detect just low velocity flow without artefacts (frequency 5 MHz; Power Doppler gain 20, range: 1-30; dynamic range: 20-40dB; pulse repetition frequency: 1 kHz). In order to assess tumor vascularisation we used the following classification: poor vascularisation – absent or isolated colour signals; abundant vascularisation – abundance of chaotic vessels in the periphery and/or the central part of the tumor (Figure 1).

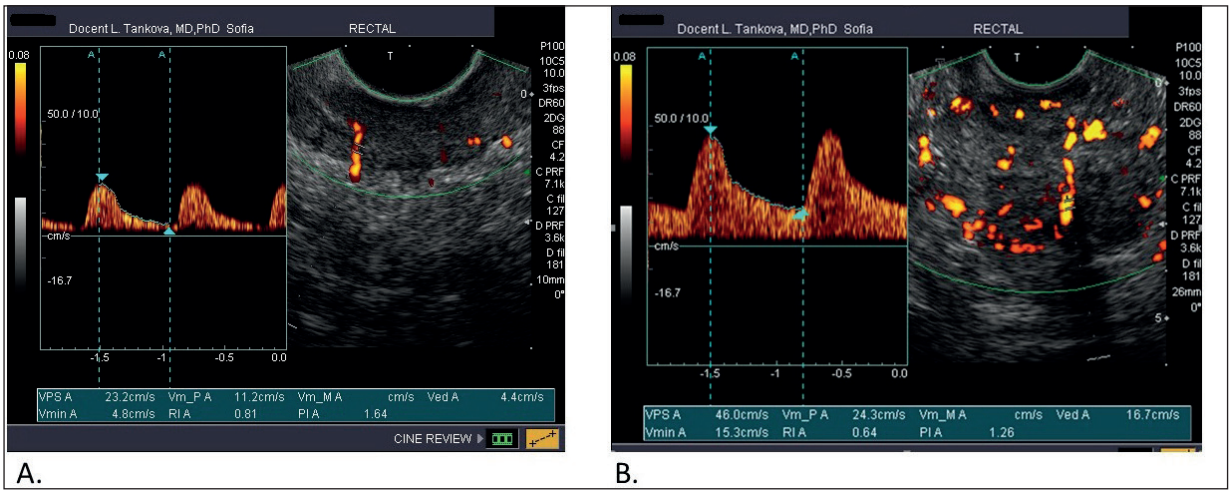

Figure 1. Power Doppler Endosonography of rectal cancer with poor vascularization ***(A)*** and high vascularization ***(B)*** – abundant chaotic vascularization in the center and periphery of the tumor.

Computer-Assisted Power Doppler Examination

In order to evaluate tumor vascularization digitally, we set a color window with Power Doppler signals including the lesion and a small area of the surrounding normal tissue. Then, three tumor slices with maximal color signal numbers were selected. After that, the tumor image was traced with a pointer, followed by a computer-assisted calculation of the percentage ratio of the number of colored pixels within a delineated tumor section to the number of total pixels in that specific tumor section. This was defined as the Power Doppler Vascularity Index (PDVI) (Figure 2)[14].

Evaluation of Angiogenesis by Immunohistochemistry

In all 110 patients, the MVD in tumor specimens was assessed. The sections were taken from the point of greatest tumor penetration into the rectal wall. The Labeled Streptavidin-Biotin 2 (LSAB2) method was applied using the Streptavidin-Biotin Peroxidase technique.

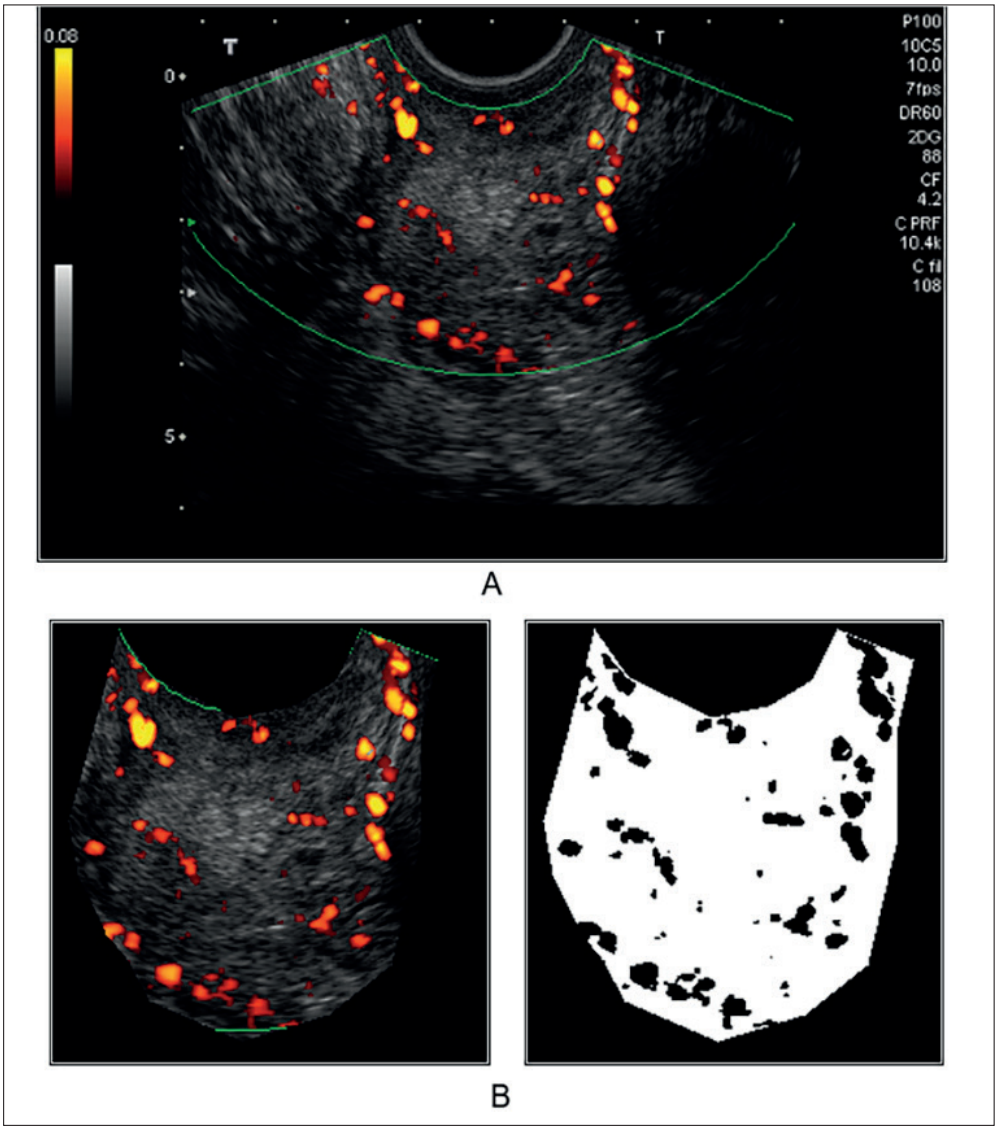

Figure 2. Power Doppler Vascularity Index for digital tumor vascularization assessment. ***(A)*** The color window is set to include the lesion and a small part of the surrounding normal tissues. ***(B)*** The tumor image is traced with the pointer, followed by a computer-assisted calculation of the percentage ratio of the number of colored pixels within a delineated tumor section to the number of total pixels in that specific tumor section.

The MVD was evaluated by using the method proposed by Weidner et al[15]. The regions with the most intensive vascularization and highest density of the brown cultured CD31+ cells (hot spots) were defined by scanning the entire tumor section at low magnification (x40 or x100) with a selection of three fields. The microvessels were counted at high magnification of either x200 (20x objective, 10x eyepiece) or x250 (25x objective, 10x eyepiece). The counting was done manually by calculating the average number of microvessels for the three selected fields and dividing this number by the size of the microscope field corresponding to each image (0.29 mm^2). Two independent pathologists counted the microvessels in the field.

Statistical Analysis

The statistical analysis was performed using SPSS for Windows, Version 25.0. (SPSS Inc., Chicago, IL, USA). For data analysis the following statistical methods were used: descriptive statistics for tabular and graphical presentation of results; Variation analysis; Student's *t*-test; Tukey's honestly significant difference (HSD) post-hoc test; One-Way ANOVA – a parametric method to test hypotheses for differences between several independent subsets; Mann-Whitney nonparametric test - to test hypotheses for differences between two independent subsets; Kaplan-Meier's method for survival curves estimation; Pearson correlation test; ROC (Receiver Operating Characteristic) curve analysis – to determine the cut-off point of the quantitative variables; Kappa coefficient for inter-rater agreement for qualitative items. The obtained results were assessed as statistically reliable within the threshold level of significance $p < 0.05$.

Ethics Approval

The study was approved by the Ethics Committee of "Tsaritsa Yoanna" University Hospital in Sofia, Bulgaria. Before initiating this study, written informed consent was obtained from all patients included in the study. The study protocol conforms to the Ethical guidelines of the 1975 Declaration of Helsinki (6th revision, 2008) as reflected in *a priori* approval by the Institution's Human Research Committee.

Results

The average size of the rectal tumors was 40.4 ± 10.4 mm. The differentiation of adenocarcinomas was as follows – well differentiated (G1) – 17 (15.6%); moderately differentiated (G2) - 78 (71.6%) and poorly differentiated (G3) – 14 (12.8%). One of the tumors was an adenosquamous carcinoma.

The mean MVD was 163 ± 69 microvessels/mm^2 (from 50 to 328). There was a very good agreement between the two pathologists (Kappa = 0.727, $p < 0.001$). In cases of disagreement, a final decision was made by consensus. The mean MVD was used as a cut-off point differentiating tumors with high (> 160 mm^2) from tumors with low (≤ 160 mm^2) angiogenic activity. There were no statistical associations observed between the MVD and the patients' age, gender or tumor size. There was a statistical significance between the MVD and the differentiation of the tumors ($p < 0.05$) (Table I). The MVD values of the poorly differentiated group were significantly higher than those of the well-differentiated group. Furthermore, the MVD had a significant association with the tumor stage ($p < 0.05$) (Table II). The mean PDVI of 8.9 ± 6.0% (0-27.3) was used as a cut-off point, dividing two groups of tumors with high (> 8%) and low (≤ 8%) PDVI. The PDVI had a significant association with the tumor stage ($p < 0.05$) (Table I), but not with the differentiation of the tumors (Table II). The PDVI was higher in advanced stages of rectal cancer. The PDVI

Table I. Analysis of statistical significance between the microvascular density (MVD) and the tumor differentiation and between the Power Doppler Vascularity Index (PDVI) and the tumor differentiation.

Tumor differentiation	n	MVD		PDVI	
		$\bar{x}$	SD	$\bar{x}$	SD
G1 - Low-grade	17	139.12[a]	45.31	7.60[a]	4.88
G2 - Intermediate grade	78	165.06[ac]	74.58	9.65[a]	6.25
G3 - High grade	14	176.71[bc]	55.52	8.45[a]	6.42

*Different letters (a, b) show a significant difference ($p < 0.05$); the same letters show no significant difference ($p > 0.05$).

Table II. Analysis of statistical significance between the microvascular density (MVD) and the rectal cancer stage and between the Power Doppler Vascularity Index (PDVI) and the rectal cancer stage.

Stage	n	MVD		PDVI	
		$\bar{x}$	SD	$\bar{x}$	SD
I	20	103.68[a]	39.83	7.29[a]	4.64
II	29	137.41[b]	48.91	7.89[a]	5.85
III	47	180.34[c]	64.84	10.29[b]	6.53
IV	14	236.07[d]	66.22	12.72[b]	5.50

*Different letters (a, b) show a significant difference ($p < 0.05$); the same letters show no significant difference ($p > 0.05$).

showed a good linear correlation with the MVD (r = 0.438, $p = 0.002$). The Kaplan-Meier analysis showed 36 months longer overall survival ($p < 0.05$) in patients with low angiogenic activity (≤ 160 mm^2 ; mean survival 66.47 months, 95% CI 60.84-72.10) than in patients with high angiogenic activity (> 160 mm^2 ; mean survival 30.37 months, 95% CI 23.52-37.22). The overall survival rate of patients with a MVD of more than 160/mm² decreased very quickly to about 30 % within a period of 30 months. Moreover, patients with a PDVI of less than 8% (mean survival 67.71 months, 95% CI 63.04-72.38) had 25 months longer overall survival ($p < 0.05$) than those with a PDVI above 8% (mean survival 42.28 months, 95% CI 35.90-48.66).

Discussion

Angiogenesis assessed by the MVD correlates with tumor behavior. Several reports[16-19] are showing that higher MVD is associated with metastases development, poor prognosis and low life expectancy in colorectal cancer patients. On the other hand, there are still conflicting results concerning the prognostic value of MVD[12,20,21].

In our study, the mean MVD is relatively high (163 microvessels/mm^2) which shows that rectal carcinoma is strongly dependent on angiogenesis. We showed that the MVD had a significant association with tumor stage and tumor differentiation.

Presumably, the high levels of MVD in the current study are because of the prevalence of advanced tumor stages in our group (57% of the tumors are in stages III and IV) and the use of CD-31 as an endothelial marker. The latter stains preexistent mature vessels, newly formed vessels, platelets, plasmocytes and megakaryocytes. Several studies show substantial differences in the MVD of carcinomas. These differences are probably related to the lack of a standardized method for tumor angiogenesis determination[22], the type of antibody used to label the endothelium and the criteria used for microvessel identification [23]. The MVD ensures direct angiogenesis evaluation and requires tumor tissue, mostly from resection specimens. However, this method is limited as it is incapable of producing information about vascular functionality, particularly in response to treatment. Indirect methods for angiogenesis determination include assessment of serum angiogenic cytokines, circulating endothelial cells and imaging methods (perfusion CT, DCE-MRI, CEUS)[5,6,24]. Endorectal ultrasonography is the standard for rectal cancer staging. In experienced hands it is quite useful and a guiding technique, even in stenotic cases and it is inexpensive compared to MRI[25]. Yimei et al [26] showed that endorectal sonography is good for early-stage patients with rectal cancer but MRI - for local advanced patients. However, less attention is generally paid to pulse colour and Power Doppler assessment. Color Doppler signals observed within the tumor depict vessels with a size of 100 μm or more in diameter - intratumoral arterioles, venules, and arteriole-venule shunts. Tumor vascularization is normally heterogeneous and chaotically allocated. The MVD evaluation in a small part of the tumor is not enough to represent the entire tumor angiogenesis and to visualize the functional properties of the tumor's blood supply. The Color Doppler enables visualization of vessels through color coding. The PDVI reflects the area of intratumoral vessels to the total tumor area and is a useful and less labor-intensive procedure for angiogenesis evaluation.

Within the current study, we demonstrate that patients with poor vascularization, determined by their PDVI, tend to live longer. Furthermore, the intensity of the intratumor angiogenesis, esti-

mated by Endorectal Power Doppler is associated with tumor stage and aggressiveness. According to our best knowledge, our study is the first that aims to correlate immunohistochemically evaluated angiogenesis and Power Doppler Sonography in rectal carcinoma. Several studies[7,8] have suggested that Color Doppler sonography may provide a useful preoperative quantitation of tumor angiogenesis and prognostic information in cancer patients. The vascular index proposed by Chen et al[27] for vascularity evaluation of rectal carcinoma during a Color Doppler Endorectal examination is a new ultrasound parameter for the study of angiogenesis. In the present study, we suggest a digital determination for the PDVI as a marker for angiogenic activity of rectal carcinoma that could facilitate patients' selection of adjuvant therapy. We show that the PDVI, by quantitatively depicting the larger supplying arterioles and draining venules, has a positive correlation with the degree of MVD in the tumor.

Conclusions

Endorectal Power Doppler Ultrasonography is a reliable and noninvasive method for assessment of the extent of rectal cancer angiogenesis. Tumor angiogenesis assessed by the PDVI correlated with histological MVD determination could predict survival rates. Endorectal Power Doppler examination is a useful and reproducible method for in vivo preoperative quantitative assessment of tumor vascularization.

Conflict of Interest

The authors received no financial support for the research, authorship, and/or publication of this article.

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